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## Exhibit M

## Research report

## Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector

Thomas J. McCown<sup>a,b,c,\*</sup>, Xiao Xiao<sup>b</sup>, Juan Li<sup>b</sup>, George R. Breese<sup>a,c,d</sup>, R. Jude Samulski<sup>b,d</sup><sup>a</sup> Brain and Development Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA<sup>b</sup> Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA<sup>c</sup> Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA<sup>d</sup> Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

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**Abstract**

Safe, long-term gene expression is a primary criteria for effective gene therapy in the brain, so studies were initiated to evaluate adeno-associated virus (AAV) vector transfer of a reporter gene into specific sites of the rat brain. In the 4 day old rat, site infusions of AAV-CMV-*lacZ* (1  $\mu$ l;  $5 \times 10^4$  particles) produced neuronal  $\beta$ -galactosidase gene expression 3 weeks later in the hippocampus and inferior colliculus, but not in the cerebral cortex. Seven days after infusion of AAV-CMV-*lacZ* viral vectors (1  $\mu$ l) in the adult rat,  $\beta$ -galactosidase gene expression was found in the olfactory tubercle, caudate, hippocampus, piriform cortex and inferior colliculus, primarily in multipolar neurons close to the infusion site. Three months after vector microinfusion, similar levels of gene expression remained in the olfactory tubercle and the inferior colliculus, with some reduction found in the caudate, but substantial reductions in  $\beta$ -galactosidase gene expression occurred in the hippocampus and piriform cortex. In no case were obvious signs of toxicity noted. Therefore, AAV vectors can transfer foreign genes into the adult and neonatal CNS, but the pattern and longevity of gene expression depends upon the area of brain being studied.

**Keywords:** Gene therapy; Viral vector; Adeno-associated virus; Inferior colliculus; Hippocampus

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**1. Introduction**

There are a number of degenerative diseases and genetic disorders where the resulting CNS pathology might be ameliorated, or even prevented, following the introduction of specific genes into the CNS. In cases where treatment would be aimed towards a degenerative disease, such as Parkinsonism, the therapeutic gene(s) must be transduced within non-mitotic cells of the adult brain. For inborn genetic diseases, the gene(s) must be introduced into the developing CNS. In either situation, long term gene transfer and expression must be accompanied by a low neurotoxic liability.

Significant advances have been made towards these goals by a number of investigators who have demonstrated successful gene transfer into the adult CNS. Palella et al. [17] showed that a herpes simplex virus type-1 (HSV-1)

vector readily transferred genetic material into CNS neurons in vivo. Although the HSV-1 vectors efficiently transfer genetic material, they are usually accompanied by substantial neurotoxicity that to date has not been overcome [16]. Adenovirus vectors also have been used to transfer genetic material into the CNS with few signs of neurotoxicity [1,8]. However, these viral vectors are episomal, thus transient, and increased titers of these vectors can cause neural damage [1]. A third viral vector system utilizes the adeno-associated virus (AAV), a widespread parvovirus that has no known associated pathology in man [2]. Using unique properties of this virus, an AAV vector composed of only *cis*-acting terminal repeats can transfer genetic material into the non-mitotic cells, but has no means of wild-type virus recombination [19]. Recently, Kaplitt et al. [7] showed that such an AAV vector could transfer an *E. coli*  $\beta$ -galactosidase (*lacZ*) gene, or a tyrosine hydroxylase gene, and produce expression of the gene product in the CNS with no obvious signs of neurotoxicity. More importantly, for up to 3 months, expression of tyrosine hydroxylase within the caudate nucleus par-

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\* Corresponding author, at: 223 Biological Sciences Research Center, Campus Box 7250, University of North Carolina, Chapel Hill, NC 27599, USA. Fax: (1) (919) 966-1844; E-mail: mccown@css.unc.edu

tially ameliorated the motor deficits caused by a prior, unilateral dopamine lesion. Even though the level of correction remained the same from 1 to 3 months, the level of tyrosine hydroxylase expression diminished somewhat over time.

These studies in the caudate nucleus established the ability of AAV-mediated gene therapy to ameliorate a degenerative CNS process, but targets of CNS gene therapy include a variety of brain sites, as well as different stages of development. Given the heterogeneity of the CNS, gene expression and longevity, as well as neurotoxicity, might vary across different brain areas. Therefore, the following studies characterized age-, time- and site-dependent gene expression patterns using the AAV vector delivery system.

## 2. Materials and methods

### 2.1. Animals

All of the animals were pathogen-free, male or female Sprague-Dawley rats obtained from Charles Rivers Associates (Raleigh, NC), or bred within the animal colony. The animals were maintained in a 12:12 h light/dark cycle and had free access to water and food. All care and procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. (NIH)85-23), and all procedures received prior approval by the IACUC at the university.

### 2.2. AAV Vector preparation

The AAV vector used in these studies was constructed by inserting the *E. coli*  $\beta$ -galactosidase gene (*lacZ*) into an AAV vector plasmid. Briefly, plasmid dl3-94 neo [14] was partially digested with restriction enzyme *Pst*I. The 2.2 kb fragment containing AAV terminal repeats and the neo gene was cloned into the *Pst*I site of plasmid pGEM-3Z (Promega), generating plasmid pdx-31. A plasmid containing the *lacZ* gene, driven by a CMV early promoter (pCMV-*lacZ*, Clontech), was digested with *Sal*I and filled in by Klenow fragment. The plasmid was subsequently digested with *Pst*I. The 4.0 kb CMV-*lacZ* fragment was gel purified and ligated with the 3.4 kb *Nsi*I-*Sna*BI fragment from pdx-31, generating AAV vector plasmid pdx31-*lacZ*.

AAV-*lacZ* viral particles were produced by cotransfecting the vector plasmid pdx31-*lacZ* with the helper plasmid AAV/AD into adenovirus-infected 293 cells [19]. Briefly, 25  $\mu$ g of plasmid DNA (6  $\mu$ g vector plus 19  $\mu$ g helper) was transfected by calcium phosphate precipitation into 293 cells at 80% confluency in DMEM plus 10% FBS. The medium was replaced after 8 to 12 h transfection with fresh DMEM plus 2% FBS. Adenovirus 5 was added to the cells at 1 m.o.i. (multiplicity of infection). After two

and a half days, the cells were harvested and freeze/thawed three times. Cell debris was removed by low-speed centrifugation. The supernatant containing AAV-*lacZ* was gently extracted 2 to 3 times with equal volume of chloroform, and the residual chloroform was evaporated with nitrogen. To the supernatant, one-third volume of saturated ammonium sulfate solution was added to make 25% saturation. The sample was placed on ice for 10 min and centrifuged at  $15,000 \times g$  for 10 min. The supernatant was recovered and saturated ammonium sulfate solution was added to make 50% saturation. The sample was placed on ice for 10 min and centrifuged at  $15,000 \times g$  for 10 min. The pellet was redissolved in cesium chloride-PBS solution (density 1.38 g/ml) and centrifuged at 40,000 rpm for 48 h. This step separates the AAV from the adenovirus. The AAV band was collected, dialyzed against DMEM and heated at 56°C for 15 to 30 min. Thus, any adenovirus will be inactivated. The AAV-*lacZ* virus titers were determined by infecting 293 cells at various dilutions. The cells were fixed and stained with X-gal, according to the methods of Hatton and Lin [5].

### 2.3. AAV Microinjection

The microinfusion of AAV-*lacZ* into 4-day-old neonatal rats utilized a modification of a previous technique [13]. In these studies, the day of birth was considered post-natal day 0, and by post-natal day 2 the litters were culled to 10 pups. Then, on post-natal day 4, the rat pups were anesthetized with ether and a 2–3 mm incision was made in the skin over the skull. Using the suture lines, rostral-caudal and medial lateral coordinates were determined, and the intersection was marked on the skull. A small hole was drilled in the skull with a 26-gauge needle, and a 33-gauge injector was lowered to the appropriate depth. After infusion of the AAV-*lacZ* vector (1  $\mu$ l over 9 min;  $5 \times 10^4$  particles/ $\mu$ l), the injector was left in place for 1 min post-infusion. The incision was sutured, and the rat pups were placed in a plexiglass cage over a heating pad. Within 2 h, the pups were returned to the dam. For the adult rats (Sprague Dawley, 45–60 days of age) microinjections of AAV-*lacZ* vectors into specific brain sites was accomplished using a previously described procedure [10]. First, the rats were anesthetized with pentobarbital (50 mg/kg) and placed into a stereotaxic frame. The skull was exposed and a hole drilled over the site to be microinjected using coordinates from the atlas of Paxinos and Watson [18]. A 33-gauge stainless steel tube was lowered into the site and 1–2  $\mu$ l of AAV-*lacZ* vectors ( $5 \times 10^4$  particles/ $\mu$ l) were infused at a rate of 1  $\mu$ l per 9 min. The injector was left in place for 1 min post-infusion to allow diffusion from the injection site. The hole was filled with bone wax, and the incision site sutured. For lateral ventricular administration, 5 or 10  $\mu$ l of the AAV-*lacZ* vector solution was infused into the lateral ventricle at a rate of 5  $\mu$ l per 9 min.

#### 2.4. $\beta$ -Galactosidase histochemistry and immunohistochemistry

On post-natal day 25, or for adults, 7 days or 3 months after AAV-*lacZ* vector infusion, animals were anesthetized with 100 mg/kg pentobarbital, i.p. and perfused transcardially with ice-cold 100 mM sodium phosphate buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4). After overnight fixation in the paraformaldehyde-phosphate buffer, vibratome sections (40  $\mu$ m thick) were taken and rinsed in PBS. For X-gal histochemistry, sections were mounted on slides and processed according to the methods of Hatton and Lin [5]. For the immunohistochemistry, tissue sections were incubated in 10% normal horse serum and 0.2% Triton X-100 in PBS for 30 min. Next, sections were incubated with a monoclonal antibody to  $\beta$ -galactosidase (1:2000 dilution; Chemicon) in 3% normal rabbit serum, 0.2% Triton X-100 and PBS for 48 to 72 h at 4°C. In some instances, adjacent sections were incubated with a monoclonal antibody to glial fibrillary acid protein (GFAP) (1:2000 dilution, Boehringer Mannheim). Tissue sections were then rinsed three times in PBS and processed through secondary biotinylated horse anti-mouse antibody and avidin-biotin complex using a Vectastain Mouse Elite ABC Kit (Vector Laboratories, Burlingame, CA). Visualization of FLI was achieved by nickel/cobalt enhancement of 3,3'-diaminobenzidine tetrahydrochloride [6].

### 3. Results

#### 3.1. AAV-*lacZ* gene transfer and expression in the hippocampus

Fig. 1 illustrates the structure of the AAV-*lacZ* vector used in the present experiments. Briefly, it can be seen that 96 percent of the AAV genome containing the replication and encapsidation genes has been removed and replaced with a *lacZ*-cytomegalovirus (CMV) promoter cassette. Thus, this viral vector does not have the ability to recombine with wild type virus. When 2  $\mu$ l of this AAV-*lacZ* vector ( $5 \times 10^4$  particles/ $\mu$ l) was infused into the hippocampus, 7 days later histochemical determination of  $\beta$ -galactosidase activity revealed reaction product localized primarily to large multipolar neurons in the hilus and pyramidal cell layers (see Fig. 2). Some reaction product also was present in the neuropil, but reaction product was noticeably absent in the dentate granule cells. Fig. 2 also illustrates the restricted spread of the AAV vector transduction, and the lack of obvious neurotoxicity. Labeled cells were found 400–500  $\mu$ m distal to the infusion site, in a rostral-caudal direction and up to 1.5 mm in a medial-lateral direction. However, neural damage was restricted to the tip of the injector and no more extensive than would be expected for a 2  $\mu$ l infusion. There were no obvious signs



Fig. 1. Schematic diagram of the AAV-*lacZ* vector. ITR stands for the inverted terminal repeat of the AAV vector. CMV stands for the early promoter from human cytomegalovirus. *lacZ* is the  $\beta$ -galactosidase gene from *E. coli*. SV splicing and SV polyA represent the SV40 splicing site and polyadenylation site, respectively.

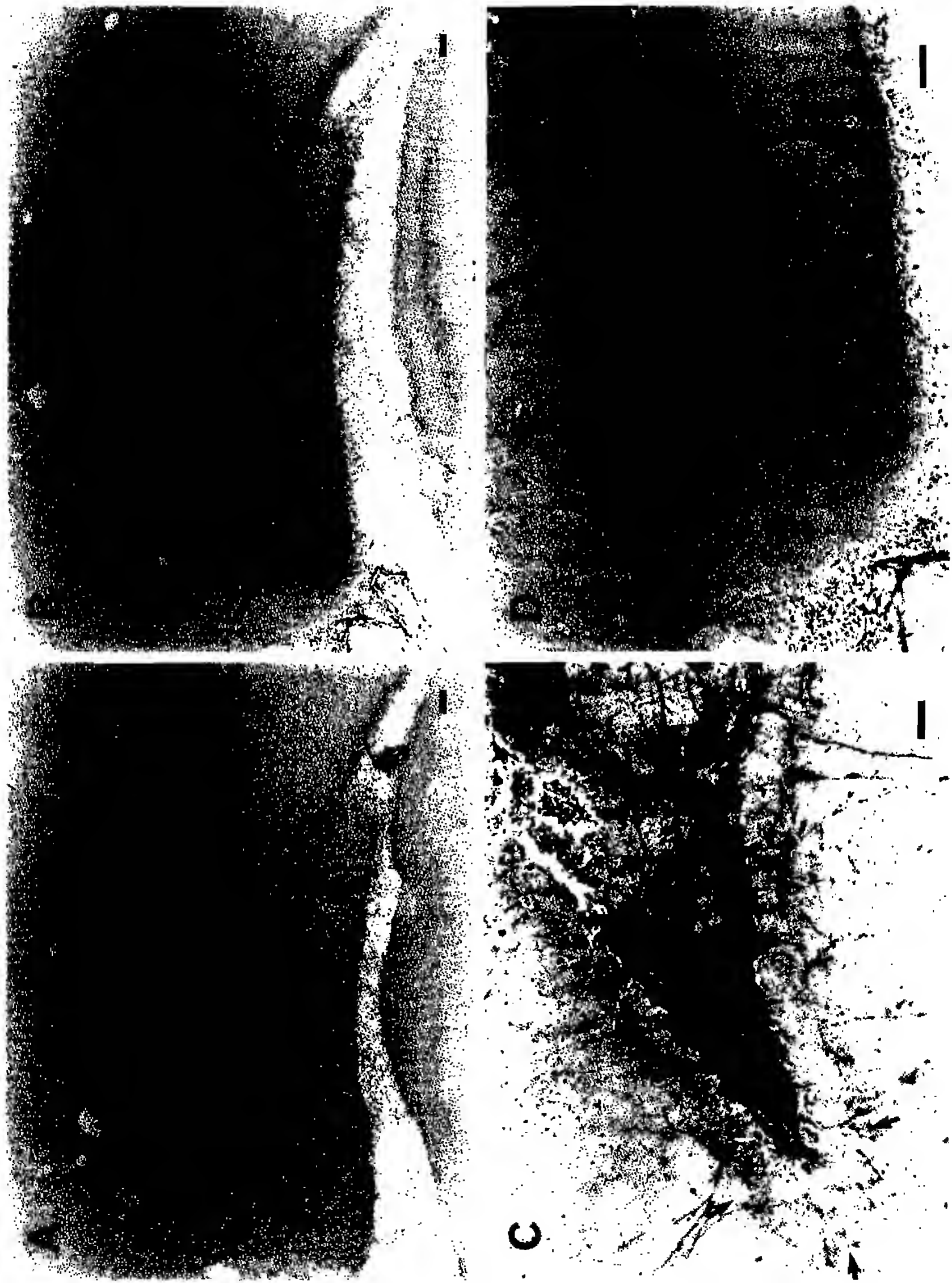
of gliosis or cell damage within areas containing gene expression.

#### 3.2. AAV-*lacZ* gene transfer and expression in the neonate

When 1  $\mu$ l of the AAV-*lacZ* vector was microinjected into different brain sites of the 4-day-old rat, a variable pattern of expression was found at 25 days of age. In the hippocampus,  $\beta$ -galactosidase immunoreactivity was found in the neuropil and large multipolar cells in close proximity to the infusion site (see Fig. 3). In contrast, no neurons exhibited  $\beta$ -galactosidase immunoreactivity after infusion into the piriform cortex of 4 neonates. When AAV-*lacZ* was infused into the inferior colliculus, many multipolar neurons exhibited  $\beta$ -galactosidase immunoreactivity (see Fig. 3), as well a few glial cells. In one animal, the injector coursed through the inferior colliculus into the adjacent central gray. As seen in Fig. 3, the remnants of a labeled dendrite comprises the extent of  $\beta$ -galactosidase immunoreactivity along the injector path in the central gray, while substantial  $\beta$ -galactosidase expression begins at the inferior collicular border and continues along the injector tract in the inferior colliculus. In all of the infusions, there were no obvious signs of damage beyond damage expected from the microinjection.

#### 3.3. AAV-*lacZ* gene transfer and expression in different brain sites of the adult at 1 week and 3 months

When 1  $\mu$ l volumes of AAV-*lacZ* vectors were infused, differential site and temporal patterns of gene expression were found. In the hippocampus, large multipolar neurons were labeled 1 week after AAV-*lacZ* infusion, but 3 months after the same infusion, the number of  $\beta$ -galactosidase positive cells was substantially reduced (see Fig. 4). Similarly, Fig. 4 shows that 1 week after the vector infusion into the piriform cortex, numerous large pyramidal cells contained  $\beta$ -galactosidase immunoreactivity, but 3 months later, both the number and extent of  $\beta$ -galactosidase positive cells was dramatically reduced. When AAV-*lacZ* vectors were infused into the caudate nucleus, some  $\beta$ -galactosidase positive cells were found 1 week later (see Fig. 4), but the number proved less than in the hippocampus or piriform cortex. Three months later, the number of  $\beta$ -galactosidase positive cells had decreased, but given the low number of positive cells at 1 week, this decrease did not appear as dramatic as that found in the hippocampus and piriform cortex.





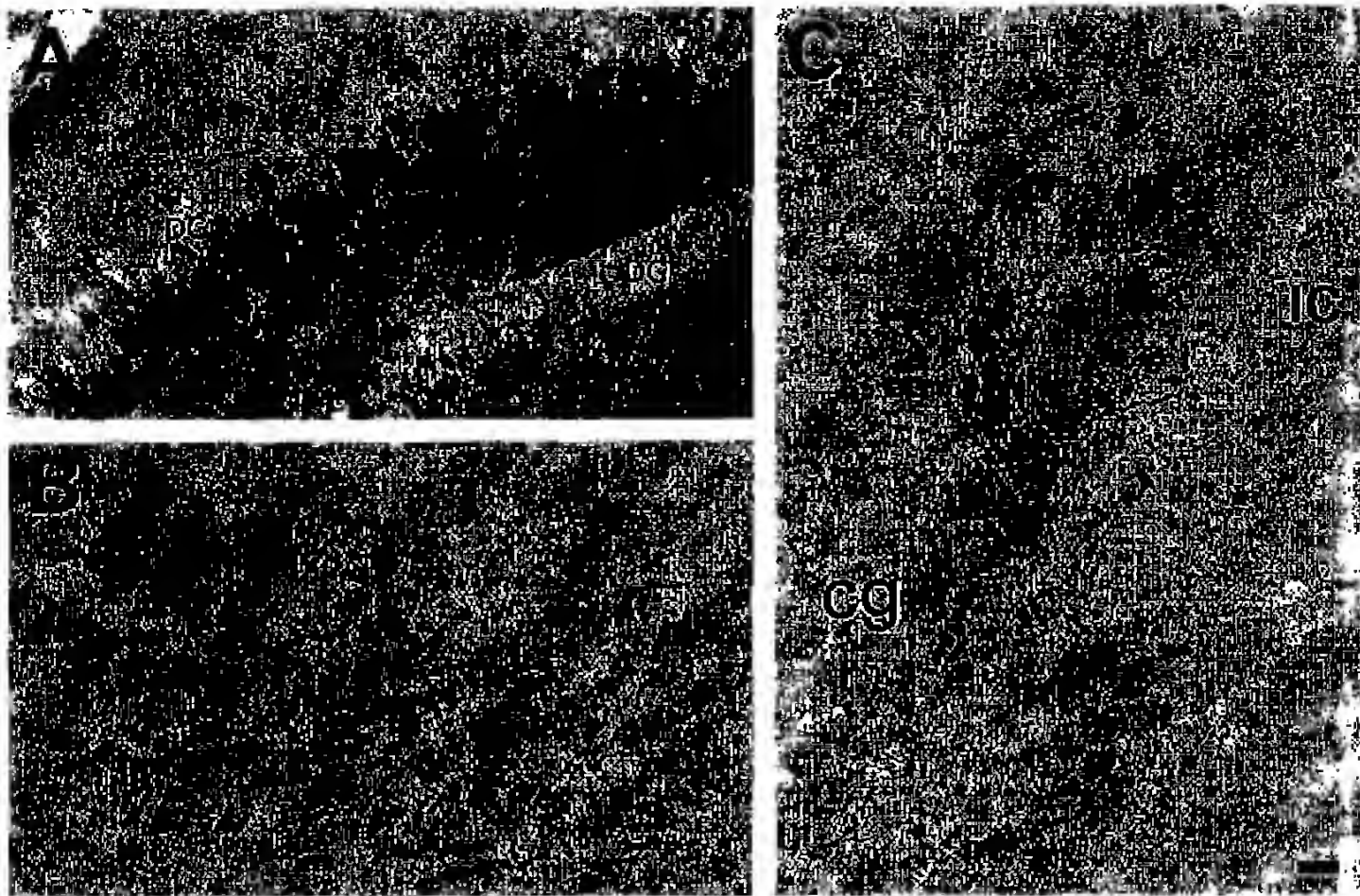


Fig. 3. The expression of  $\beta$ -galactosidase 3 weeks after the infusion of 1  $\mu$ l of AAV-*lacZ* vectors ( $5 \times 10^4$  particles/ $\mu$ l) into the hippocampus (A) or inferior colliculus (B) of the 4-day-old rat. In both the hippocampus and the inferior colliculus, multipolar neurons comprise the majority of the cells labeled for  $\beta$ -galactosidase immunoreactivity. In panel C, the injector coursed through the inferior colliculus and into the central gray. A dendritic remnant remains labeled near the tip of the injector (\*), but little expression occurs until the border of the inferior colliculus, where substantial  $\beta$ -galactosidase immunoreactivity is found. The large labeled cells demarcate the border between these adjacent brain structures. DG, dentate granule cell layer of the hippocampus; ic, inferior colliculus; cg, central gray. Bar = 50  $\mu$ m.

In direct contrast, two brain areas exhibited similar amounts of gene transfer and expression both at 1 week and 3 months. As seen in Fig. 5, AAV-*lacZ* vector infusion into the olfactory tubercle resulted in a moderate number of  $\beta$ -galactosidase positive multipolar neurons at 1 week. Similar numbers of labeled, multipolar neurons were found 3 months after AAV-*lacZ* vector infusion. When AAV-*lacZ* vectors were infused into the inferior colliculus, a substantial number of  $\beta$ -galactosidase positive neurons were present both at 1 week and 3 months (see Fig. 5). Even though the inferior colliculus exhibits a high level of long-term transduction, GFAP immunohistochemistry in adjacent sections did not reveal obvious signs of glial reactivity (see Fig. 6).

In all of these brain areas,  $\beta$ -galactosidase immunoreactivity was restricted to the immediate vicinity of the infusion, occurring primarily in neurons. However, there were a few instances where  $\beta$ -galactosidase immuno-

reactivity was found in a clearly identified astrocyte. Also, when 5 or 10  $\mu$ l of the AAV-*lacZ* vectors were infused into the lateral ventricle, 7 days later  $\beta$ -galactosidase was found in ependymal cells close to the site of infusion, but not in adjacent brain structures.

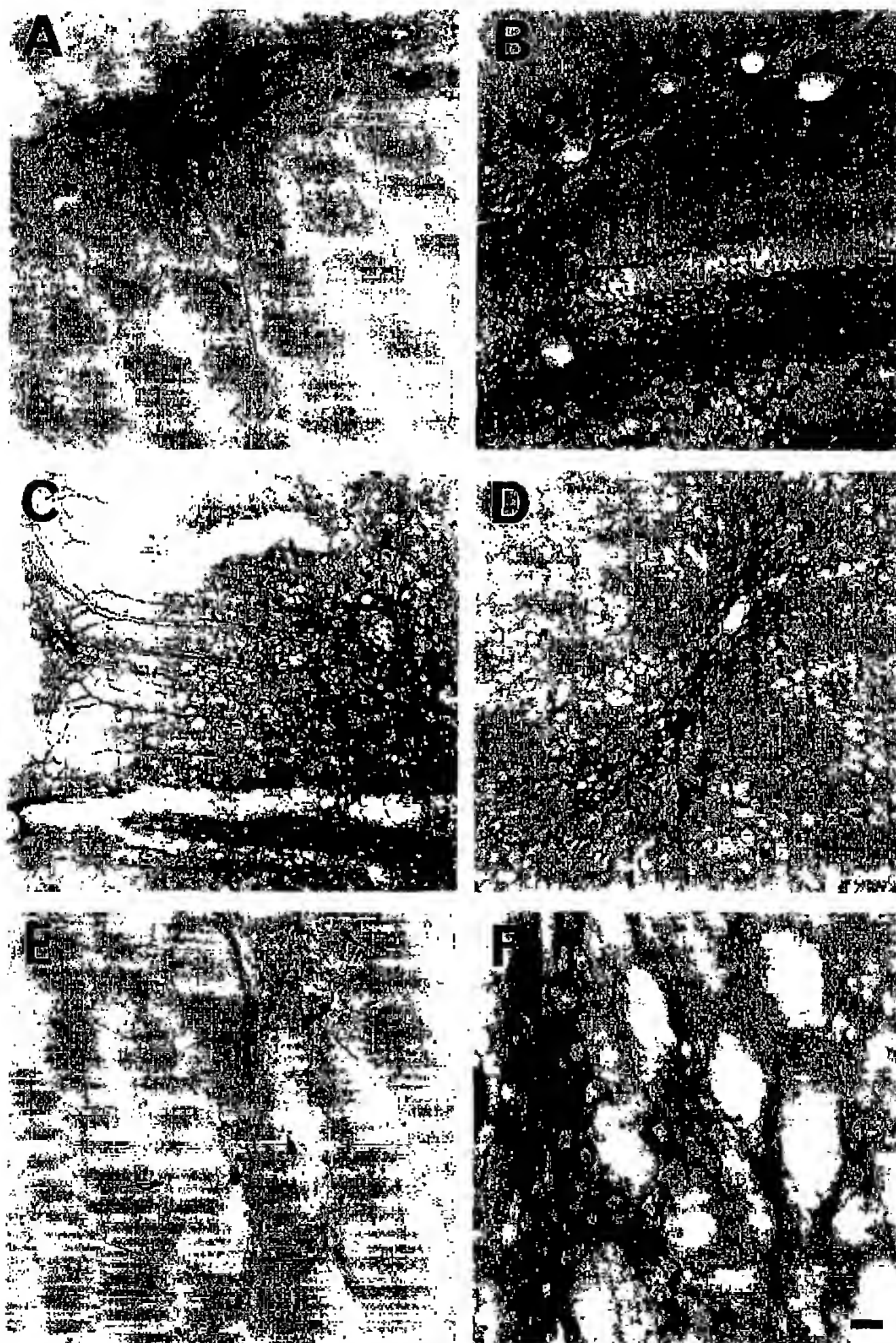
#### 4. Discussion

When AAV-*lacZ*-CMV promoter vectors are microinfused into the brain, 15 to 40  $\mu$ m diameter neurons represent the major cell type where transfer and expression of  $\beta$ -galactosidase occurs. However, different levels of gene transfer and expression were found both across and within different brain regions. After a 1  $\mu$ l infusion, the caudate nucleus exhibited the fewest number of labeled cells, never more than 10 cells in a given section. A greater number of cells were found in the olfactory tuber-

Fig. 2. The expression of  $\beta$ -galactosidase activity in adult hippocampus 7 days after a 2  $\mu$ l infusion of AAV-*lacZ* vectors ( $5 \times 10^4$  particles/ $\mu$ l) using X-gal histochemistry in cresyl violet-stained 40- $\mu$ m thick sections. In panel A the large arrow demarcates the tip of the injector, while the blue reaction product illustrates the extent of the  $\beta$ -galactosidase expression. Upon enlargement (panel C), notice that large multipolar neurons in the hilus of the dentate gyrus, dendrites and some neuropil exhibit  $\beta$ -galactosidase activity. However, no labeling is present in the dentate granule cell layer, outlined by the small arrows. Panel B represents a section 400  $\mu$ m distal to the injection site. Again most labeling occurs in multipolar neurons, dendrites and neuropil, but no labeling is found in the dentate granule cells. In the enlargement (D), both large multipolar neurons, as well as the outline of the dentate granule cell layer can be seen. Bar = 50  $\mu$ m.

cle, between 10 and 20 cells in a given section. The greatest number of labeled cells were generally found in the piriform cortex, hippocampus and inferior colliculus. Usually, sections from these areas exhibited more than 20 labeled cells per section, while in the case of the inferior colliculus, some sections had greater than 50  $\beta$ -galactosidase positive cells in a given section (see Fig. 5). How-

ever, within given brain regions, not all neurons exhibited  $\beta$ -galactosidase expression. For example, while many multipolar neurons were  $\beta$ -galactosidase positive in the hippocampus,  $\beta$ -galactosidase activity was noticeably absent in the dentate granule neurons (see Fig. 2). Also, there were instances where other cells types exhibited gene transfer and expression. After ventricular administration,





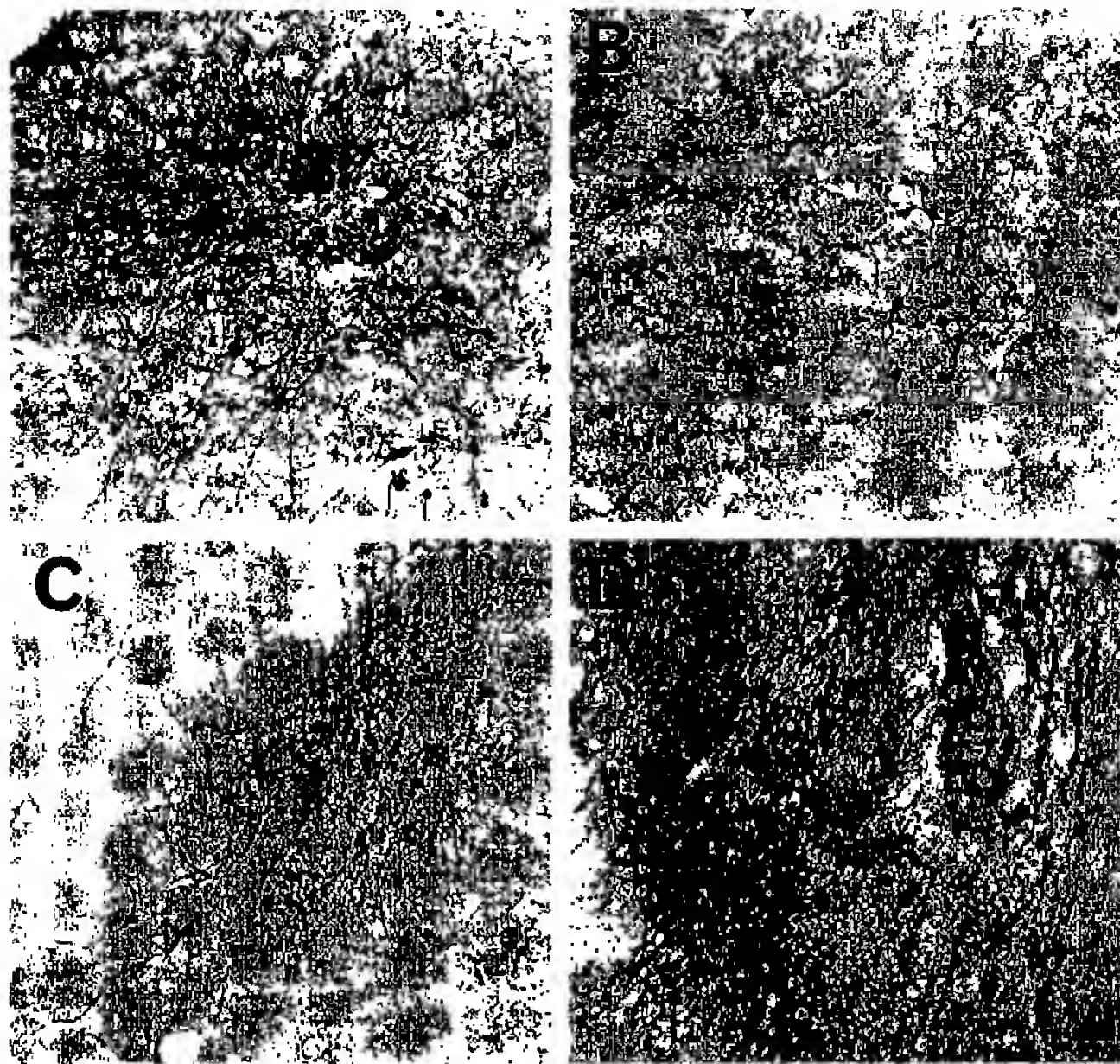


Fig. 5. The expression of  $\beta$ -galactosidase in adult inferior colliculus and olfactory tubercle 7 days and 3 months after a  $1 \mu\text{l}$  infusion of AAV-*lacZ* ( $5 \times 10^4$  particles/ $\mu\text{l}$ ). For the inferior colliculus, numerous neurons exhibited  $\beta$ -galactosidase immunoreactivity both 7 days (A) and 3 months (B) after AAV vector infusion. These findings were consistent for the 7-day ( $n = 5$ ) and 3-month ( $n = 3$ ) groups. Similarly, in the olfactory tubercle, a moderate number of neurons exhibited  $\beta$ -galactosidase immunoreactivity both 7 days (C) and 3 months (D) after AAV vector infusion. Likewise, these findings were consistent for the 7-day ( $n = 4$ ) and 3-month ( $n = 3$ ) groups. Bar =  $50 \mu\text{m}$ .

ependymal cells lining the ventricles were positive for  $\beta$ -galactosidase, and occasionally, glia did exhibit expression of  $\beta$ -galactosidase. In the case of astrocytes, the relative lack of labeling does not appear to be a problem with detection sensitivity, because in one case, a labeled astrocyte was clearly identified in the caudate nucleus. Although expression appears to favor large multipolar neurons, this observation does not mean that the AAV vectors are only taken up into multipolar neurons or that

gene transfer is more efficient in neurons. It only means that with the AAV vector under control of the CMV promoter, expression of the gene product occurs preferentially in multipolar neurons. Given a other vectors or promoters, the pattern of gene transfer could be quite different. For example, Le Gal La Salle et al. [8] found  $\beta$ -galactosidase expression in the dentate granule cells, but not the hilus or pyramidal cell layer, after hippocampal infusion of an adenovirus vector driven by a Rous sarcoma

Fig. 4. The expression of  $\beta$ -galactosidase in adult hippocampus, piriform cortex and caudate nucleus 7 days and 3 months after a  $1 \mu\text{l}$  infusion of AAV-*lacZ* ( $5 \times 10^4$  particles/ $\mu\text{l}$ ). In the hippocampus, a number of large multipolar neurons are labeled in the pyramidal cell region of CA1(A; \* = injector tip), but 3 months after an infusion into the same area (B; \* = injector tip), only one neuron exhibits  $\beta$ -galactosidase immunoreactivity. For the 7-day survival group, there were at least 10 labeled cells per section ( $n = 5$ ), while the 3-month survival group never exhibited more than 5 labeled cells in a section ( $n = 3$ ). Panels C and D show the 7-day and 3-month post-infusion results respectively for the piriform cortex. At 7 days, substantial labeling was found primarily in pyramidal cells and their dendrites (C), but by 3 months after the AAV vector infusion (D), only a few cells exhibited any  $\beta$ -galactosidase immunoreactivity near the tip of the injector (\*). Again, the 7-day survival group exhibited greater than 20 cells per section ( $n = 4$ ), while the 30-day survival never exhibited greater than 10 cells per section or any dendritic labeling ( $n = 3$ ). In the caudate nucleus, some multipolar neurons are labeled in close proximity to the injector tract (E), but by 3 months fewer neurons were positive for  $\beta$ -galactosidase immunoreactivity around the injector tract (F). In the 7-day group ( $n = 5$ ), there were never more than 10 positive cells in a given section, while in the 3-month group, there were never more than 5 positive cells in a given section ( $n = 3$ ). Bar =  $50 \mu\text{m}$ .



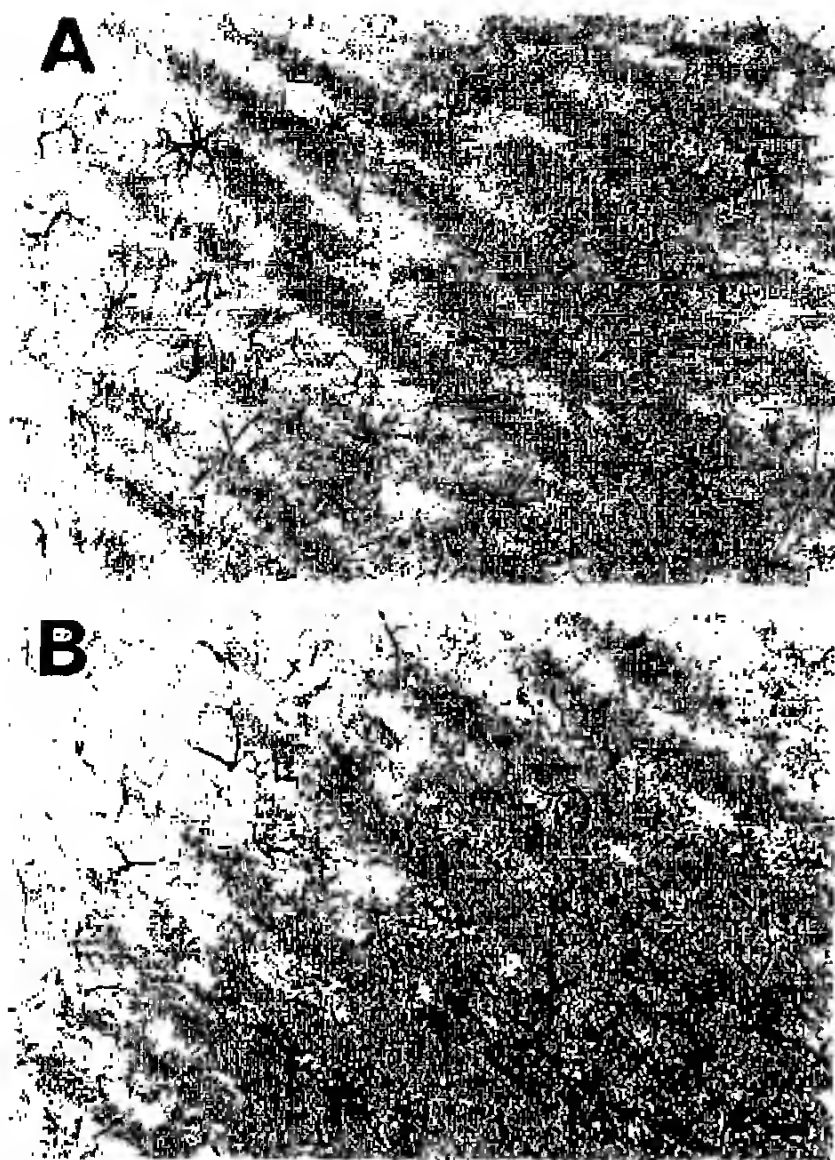


Fig. 6. GFAP immunoreactivity in sections from the inferior colliculus 3 months after the injection of 1  $\mu$ l AAV-lacZ ( $5 \times 10^4$  particles/ $\mu$ l). It can be seen that the pattern of astrocyte GFAP immunoreactivity in the section contralateral to the AAV vector infusion (A) does not appreciably differ from the GFAP immunoreactivity on the infused side (B). The adjacent section to B exhibited  $\beta$ -galactosidase immunoreactivity similar to that found in Fig. 5, panel B. Bar = 50  $\mu$ m.

virus long terminal repeat promoter. Further investigations will be necessary to delineate any cell specific differences in the AAV-vector uptake, gene transfer or promoter driven gene expression.

These studies also showed that AAV vectors can effectively transfer foreign genes into the neonatal brain, but the expression of  $\beta$ -galactosidase proved to be site specific. Substantial  $\beta$ -galactosidase expression was found in the hippocampus and inferior colliculus, but not in the piriform cortex. One explanation for this difference could be the stage of brain development. For example, the lack of neural labeling in the cortex might be attributed to the fact that in 4-day-old rats, pyramidal cells are still migrating along the radial glia towards their final destination [15]. Alternatively, there could be site-specific differences in promoter regulation. Clearly, dramatic gene expression differences were found between two adjacent structures, the central gray and the inferior colliculus. Thus, in the neonate, the pattern of gene expression likely depends upon both differences in promoter regulation, as well as

the stage of development. Again, future studies will be necessary to define the actual contribution of these processes to gene transfer and expression in the neonatal brain.

In the adult rat, AAV vectors are capable of long-term transfer and expression of a foreign gene without overt signs of neurotoxicity, but the longevity of expression is brain site specific. In the piriform cortex and the hippocampus, the number of cells expressing  $\beta$ -galactosidase at 3 months was dramatically lower than the number found 7 days post-infusion. In the caudate nucleus, the amount of expression at 7 days was not as high as in the cortex or hippocampus, but there still appeared to be some decline in the number of  $\beta$ -galactosidase-positive cells at 3 months. However, it must be noted that even with such a decline in expression, Kaplitt et al. [7] found that AAV transfer and expression of tyrosine hydroxylase in the caudate was sufficient to partially reverse a lesion-induced motor deficit. Thus, for some disorders, partial long-term expression may be therapeutically sufficient. In marked contrast, both the olfactory tubercle and the inferior colliculus exhibited the same relative amount of  $\beta$ -galactosidase expression at 7 days and 3 months. Furthermore, in the inferior colliculus, there were no obvious signs of reactive astrocytes, so AAV-mediated long term gene expression did not produce obvious signs of neurotoxicity.

These site-specific differences in long-term gene expression could be attributed to variable expulsion of the vector or differential regulation of the CMV promoter. Given the difficulties with finding promoters capable of long term expression [4], and recent findings by Bloom et al. [3], it seems likely that inactivation of the promoter underlies our observed decline in gene product expression. For example, Bloom et al. [3] recently demonstrated long-term expression of  $\beta$ -galactosidase in the hippocampus using an HSV vector and a latency associated transcript promoter from murine moloney leukemia virus. However, the 6-month level of gene expression was substantially less than the levels of expression at 2 weeks, even though the amount of viral DNA did not change over this time period. In the present studies, differential suppression of the CMV promoter would explain why some brain areas exhibited a long term decline in  $\beta$ -galactosidase expression, while other brain areas showed no decline in  $\beta$ -galactosidase expression. Clearly, additional studies will be necessary to confirm this supposition.

In conclusion, these studies show that an AAV-lacZ vector can transfer genetic material into neurons of the CNS and in some brain regions, expression of the gene product remains for at least 3 months without obvious signs of neurotoxicity. In other brain regions, the expression of the gene product decays over time. Thus, findings on viral vector gene transfer in one brain area may not generalize to other brain areas. Just as important, with the AAV vector and the CMV promoter, both neonatal and long-term adult expression of  $\beta$ -galactosidase was found

in the inferior colliculus. Since neonatal and adult seizure genesis has been well characterized in the cortical subdivision of the inferior colliculus [9,11–13], this brain area should prove to be excellent model system to explore various strategies of gene therapy.

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